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(54) Title: MAST CELL REGULATION

(57) Abstract: The present invention relates to the use of a compound capable of suppressing the expression of the human bfl-1 gene or a homologue thereof in the manufacture of a medicament for treating a mast cell mediated inflammatory disorder in a mammal, most preferably a human, by negatively regulating the longevity and/or activity of activated mast cells. In an alternative embodiment, a compound capable of suppressing the function of an expression product, such as an mRNA or a protein, is used for said purpose. The invention also relates to such a medicament as such as well as to methods for identifying compounds useful in such medicaments.

MAST CELL REGULATION

Field of invention

The present invention relates to the manufacture of a novel medicament for the treatment of a mast cell mediated inflammatory disorder in a mammal, most preferably a human. In addition, the present invention also relates to various methods for the identification of compounds useful as active ingredients in such medicaments as well as to a pharmaceutical composition.

Background of the invention

The occurrence of allergic diseases has increased during the last decades, especially in the Western world. Most allergic diseases are caused by IgE-mediated (immunoglobulin E) hypersensitivity reactions. Upon activation, during an allergic reaction, IgE molecules bind via a cell-binding constant domain to specific receptors (FcεRI) on mast cells.

Mast cells are generally considered to be long living cells, that are widely distributed throughout vascularized tissues and certain epithelia where they play a fundemental role in the pathogenesis of immediate hypersensitivity reactions. In inflammatory disorders, such as allergies and asthma, increased numbers of mast cells in affected tissues have been documented with a positive correlation between mast cell number and the severity of the allergic response symptoms. [Otsuka, H., J. Denburg, J. Dolovich, D. Hitch, P. Lapp, R.S. Rajan, J. Bienenstock, and D. Befus. 1985. Heterogeneity of metachromatic cells in human nose: Significance of mucosal mast cells. *J. Allergy Clin. Immunol.* 76:695-702].

Upon activation and FcεRI aggregation, mast cells release preformed mediators such as histamine, proteases, tryptase and heparin, and synthesize lipid mediators and cytokines causing the allergic response observed in sensitized individuals. Survival, growth and differentiation of mast cells are regulated by cytokines such as interleukin-3 (IL-3) and stem cell factor (SCF). Mast cells have the capacity to survive the activation-induced degranulation process, and to subsequently regranulate,

enabling them to be activated again. Multiple rounds of mast cell activation may underlie the mechanism regulating the reoccurring inflammatory attacks of allergic patients during a pollen season. The possibility of regulating the longevity of activated mast cells could thus provide a treatment for mast cell mediated inflammatory disorders, such as allergies and asthma. However, the molecular mechanisms enabling activated mast cells to survive have not been elucidated.

A well documented mechanism for regulating cell longevity and survival is through the up-regulation of pro-survival genes belonging to the bcl-2 protein family. This is a growing family of apoptosis-regulatory genes, which may either be death antagonists (bcl-2, bcl-XL, bcl-w, mcl-1 and A1) or death agonists (bax, bak, bcl-XS, bad, bid, bik and hrk) [Reed, J. 1994. Bcl-2 and the regulation of programmed cell death. *J. Cell Biol* 124:1-6]. Although the precise mechanism by which bcl-2 family members influence apoptosis is unknown, several lines of evidence suggest that bcl-2 proteins function at a critical decision point immediate upstream of an irreversible commitment to cell death [Dragovich, T., C. M. Rudin, and C. B. Thompson. 1998 Signal transduction pathways that regulate cell survival and cell death. *Oncogene* 17:2107-13.]. It has been demonstrated that nerve growth factor (NGF) markedly increases bcl-2 expression in mast cells. When overexpressed, bcl-2- prolongs survival of mast cells following IL-3 deprivation. SCF and IL-3 induce suppression of apoptosis in mast cells by different mechanisms since IL-3 can induce bcl-2 induction but SCF cannot [Yee, N. S., I. Peak and P. Besmer. 1994. Role of the c-kit ligand in proliferation and suppression of apoptisis in mast cells. Basis for radiosensitivity of white spotting and steel mutants. *J. Exp. Med.* 179:1777-1787.]. Despite the wide-ranging ability of bcl-2 to promote cell survival, there are circumstances where bcl-2 is not found to be responsible for protecting the cells from apoptosis [Hueber, A., G. Raposo, M. Pierres and H. He. 1994. Thy-1- triggers mouse thymocyte apoptosis through a bcl-2-resistant mechanism. *J Exp Med* 179:785-96.]. Therefore, it is possible that other members of the bcl-2 family may provide protective effects in some specific biological processes. In monocytes IgE-receptor aggregation leads to up regulation of bcl-X_L and bcl-2 [N. Katoh, S. Kraft, J. H. M. Wes-

sendorf, T. Bieber. 2000. The high-affinity IgE receptor (FcεRI) blocks apoptisis in normal human mononcytes. *J. Clin.Invest.* 105: 183-190].

A1 was originally identified from mouse bone marrow cultured cells induced with granulocytemacrophage-colony stimulating factor (GM-CSF) and bears structural homology to bcl-2 [Lin,E., A. Orlofsky, M. Berger and M. Prystowsky. 1993. Characterization of A1, a novel hemopoietic-specific early-response gene with sequence similarity to bcl-2. *J. Immunol* 151:1979-88.]. A1 was described as an early-response gene, expressed in multiple tissues such as thymus, spleen and bone marrow, and also expressed in a number of hemotopoietic cell lineages under various stimulations. These cells include T and B lymphocytes, macrophages, neutrophills [Chuang, P. I., E. Yee, A.Karsan, R.K. Winn and J. M. Harlan. 1998. A1 is a constitutive and inducible Bcl-2 homologue in mature human neutrophils. *Biochem. Biophys. Res. Commun.* 249:361-5], and endothelial cells [Lin,1993, supra]. A1 or the human homologue bfl-1 [S.S. Choi, S.H. Park, U. –J. Kim, H.-S. Shin.1997. Bfl-1, a Bcl-2-related gene, is the human homolog of the murine A1 and maps to Chromosome 15q24.3. *Mammalian Genome* 8, 781-782; Choi S.S., Park I.;., Yun J.W., Sung Y.C., Hong S.I., Shin H.S. 1995. A novel Bcl-2 related gene, Bfl-1 is overexpressed in stomach cancer and preferentially expressed in bone marrow. *Oncogene* 11(9):1693-8] is the only bcl-2 family member that is inducible by inflammatory cytokines such as tumor necrosis factor-α and IL-1β.

Available drugs for allergic diseases treat the symptoms of the disease which occurs as a consequence of released mediators from inflammatory cells. A treatment that more specifically would prevent the allergic response, with reduced side effects, compared to current drugs, would be desirable.

Summary of the present invention

Accordingly, one object of the present invention is to provide a method for the treatment of mast cell mediated inflammation which instead of treating the symptoms enables control of the disease on a gene regulatory level. This is achieved according

to the invention by the use of a compound capable of suppressing or even inhibiting the expression of the human bfl-1 gene or a homologue thereof, such as the mouse homologue A1, in the manufacture of a medicament for treating a mast cell mediated inflammatory disorder in a mammal, most preferably a human, by negatively regulating the longevity and/or activity of activated mast cells.

Another object of the present invention is to enable treatment of mast cell mediated inflammation on a protein level. This is achieved by using a compound capable of suppressing or even inhibiting the function of an expression product of the human bfl-1 gene or a homologue thereof, such as the mouse homologue A1, in the manufacture of a medicament for the above discussed purpose.

A further object of the invention is to provide a method for identifying a novel compound useful for the treatment of a mast cell mediated inflammatory disorder. Such a method can e.g. be a chemical or biological assay, as will be discussed in more detail below. Further objects of the present invention are achieved as disclosed in the appended claims.

Brief description of the drawings

Figure 1A-C illustrate survival promotion of murine mast cells after cross-linkage of FcεRI (IgE CL).

Figure 2 illustrates the survival promotion of human mast cells after cross-linkage of FcεRI (IgE CL).

Figure 3A-B illustrate bcl-2 family gene regulation in mouse mast cells after cross-linkage of FcεRI (IgE CL).

Figure 4 A-B illustrate bcl-2 family gene regulation in human mast cells after cross-linkage of FcεRI (IgE CL).

Figure 5 illustrates the time-dependent up-regulation of the A1 gene.

Figure 6 A-B illustrate regranulation of mouse mast cells.

Figure 7 A-B illustrate the effects of ionomycin (A) and compound 48/80 (B) on A1 induction, release and survival of MCP5/L cells.

Figure 8 A-C illustrate the effects of cold incubation and EDTA treatment on A1 induction and mast cell degranulation.

Figure 9 A-B illustrate the effects of inhibitors on induction of A1 after activation through FcεRI cross-linkage.

Figure 10 illustrates the effect of cyclosporin A (CSA) on the survival promotion of mast cells through FcεRI cross-linkage

Figure 11 A-B illustrate the absence of survival promotion of mast cells from $A1^{-/-}$ mice after FcεRI cross-linkage (IgE CL), although release. β-hexosaminidase (β-hex)comparable to mast cells from wild type mice.

Figure 12 illustrate the induction of A1 protein in moue mast cells after cross-linkage of FcεRI.

Figure 13 A-B illustrate the the effect of dexametasone (DEX), Piceatannol (Pic), Wortmannin (WTN) and EDTA on the induction of bfl-1 in human mast cells after cross-linkage of FcεRI.

Figure 14 A-B illustrate that cross-linkage of FcεRI but not an other mast cell secretagogoue, adenosine, induce expression of A1 and promotes mast cell survival.

Detailed description of the invention

In a first aspect, the present invention relates to the use of a compound capable of suppressing or essentially inhibiting the expression of the human bfl-1 gene or a homologue thereof in the manufacture of a medicament for treating a mast cell mediated inflammatory disorder in a mammal, most preferably a human, by negatively regulating the longevity and/or activity of activated mast cells.

In the present context, it is to be understood that the term "bfl-1 homologue" is intended to include genes from other species which exhibits a pro-survival or anti-apoptotic function equivalent to that of the bfl-1 gene. Suppression of other variants e.g. caused by degeneration of the genetic code, mutation, splice variants etc, which fulfill such a function in a cell, are also encompassed within the scope of the invention.

In the present context, it is to be understood that suppression of the expression of a gene can be obtained by any suitable method, and includes e.g. suppressing the transcription by blocking the regulatory elements, e.g. the promoter elements, or suppressing the translation by blocking the messenger RNA, e.g. through an antisense treatment.

In one embodiment, the present invention relates to the use of a compound capable of suppressing or essentially inhibiting the expression of a gene for the above discussed purpose, the messenger RNA and protein encoding region of which comprises at least about 95% of the mRNA and protein encoding region of the nucleic acid sequence as disclosed in SEQ ID NO. 1. In a specific embodiment, the gene which is suppressed or inhibited according to the invention comprises at least about 97%, preferably at least about 98% and most preferably at least about 99% of the mRNA and protein encoding region of SEQ ID NO 1. However, as appears from the above, the actual regulation or suppression of the gene in accordance to the invention does not necessarily need to occur within such specified regions, as long as it results in a suppression of the expression of such a specified sequence.

In a specific embodiment, the present invention relates to the use of a compound capable of suppressing or essentially inhibiting the expression of a murine homologue to the bfl-1 gene for the above discussed purpose. Such murine homologues are commonly denoted the A1 gene family, the open reading frame sequences of which exhibit an identity of about 97%, as determined in accordance with Hatakeyama et al, 1998 [Hatakeyama, S., A. Hamasaki, I. Negishi, D.Y. Loh, F. Sendo, K. Nakayama, and K.-i., Nakayama. 1998. Multiple gene duplication and expression of mouse bcl-2-related genes, A1. *Int. Immunol.* 10:631-637].

Thus, according to the invention, it has surprisingly been found that by inhibiting the anti-apoptotic function of bfl-1 or A1 gene or gene product, such as the mRNA or protein, the activated mast cells undergo apoptosis, preventing re-granulation and re-activation. In that way the number of activated mast cells in the inflammatory

tissue can be reduced and the allergic symptoms alleviated. The present invention provides a means to selectively target and eliminate mast cells that are actively involved in mediating the inflammatory response. Thus, as the skilled in this field will realise, this desired result can be achieved by a regulation on any one of three levels, namely on a gene or DNA level; on a transcription (mRNA) level; or on a translational (protein) level. Consequently, in the present context, it is to be understood that the terms "gene product" and "expression product" are used interchangeably and include both the expression product on a transcriptional level i.e. the mRNA and the expression product on a translational level i.e. the protein. On an mRNA level, the term "expression product" is intended herein to include any homologue or variant of the bfl-1 mRNA. On a protein level, the term "expression product" of the above discussed bfl-1 gene, or any analogue, variant or functional fragment or derivative of such a product.

Accordingly, in a second aspect, the present invention relates to the use of a compound capable of suppressing or essentially inhibiting the function of an expression product of the human bfl-1 gene or any homologue thereof in the manufacture of a medicament for treating a mast cell mediated inflammatory disorder in a mammal, most preferably a human, by negatively regulating the longevity and/or activity of activated mast cells. The function of such an expression product consequently refers to its biological function based on its anti-apoptotic, pro-survival properties.

In one embodiment, such an expression product is a transcription product, i.e. an mRNA. In a preferred embodiment, the suppression is provided by the use of antisense technology.

In another embodiment, said expression product is a translation product of the human bfl-1 gene or a homologue thereof. In a specific embodiment, the compound used is capable of suppressing or essentially inhibiting the function of a protein which comprises at least about 95% of the amino acid sequence disclosed in SEQ ID NO. 2, such as at least about 97%, preferably at least about 98% and most prefe-

rably at least about 99% of SEQ ID NO. 2. Thus, the present invention also encompasses the use of any variant or functional fragment of the bfl-1 protein, which is capable of exerting an equivalent biological function as the human bfl-1, as discussed above.

In another embodiment, the compound used is capable of suppressing or essentially inhibiting the function of an expression product of a bfl-1 homologue of another species, such as the murine homologue A1, as discussed above. At the amino acid level, the A1 gene family exhibits an identity of about 96%, as determined in accordance with Hatakeyama et al, 1998.

In a specific embodiment, the compound used according to the invention is a compound capable of inhibiting the bfl-1 or A1 dependent survival of activated mast cells.

The level of suppression obtained by a compound can easily be tested by methods well known in this field, e.g. as disclosed below in the Experimental section.

In one embodiment, the present compound is a (poly)peptide, an oligonucleotide or any other organic molecule. In a specific embodiment, the compound is selected from the group consisting of cyclosporin A, piceatannol, a PI-3 kinase inhibitor, such as wortmannin, or a glucocorticoid, such as dexamethasone.

The present invention also encompasses a pharmaceutical composition manufactured as described above. Thus, in another aspect, the present invention relates to a pharmaceutical composition which comprises a compound capable of suppressing or essentially inhibiting the expression of a human bfl-1 gene or a variant thereof, or a homologue or variant thereof, for the above discussed purpose in a therapeutically effective dose together with a pharmaceutically acceptable carrier. In an alternative aspect, the invention relates to a pharmaceutical composition which comprises a compound capable of suppressing or essentially inhibiting the function of an ex-

pression product of any one of the above discussed genes, such as an mRNA or a protein, in a therapeutically effective dose together with a pharmaceutically acceptable carrier.

In a further aspect, the invention relates to a biological assay for identifying a compound useful in the treatment of a mast cell mediated inflammatory disorder in a mammal, most preferably a human. In an illustrating biological assay, mast cells are cultured under serum-free conditions without addition of any cytokines, growth factors or other potential mast cell survival factors. The cells are then activated either through the high affinity IgE-receptor or by other mast cell secretagogoues. Mast cell viability and/or apoptosis is then determined over the next following days, and the expression of A1 is determined on either mRNA or protein level, e.g. as described below in the experimental section.

In another aspect, the invention relates to a chemical assay for identifying a compound useful in the treatment of a mast cell mediated inflammatory disorder in a mammal, most preferably a human. The assay comprises using the anti-apoptotic bfl-1/A1 gene or gene product, such as mRNA or protein, as a molecular target. Such a chemical assay can e.g. be based on a downstream regulation of the gene, it can be a binding assay or any other suitable format the skilled person in this field would envisage based on the novel findings according to the invention as described above and in the experimental part of this application.

In another aspect of the invention, the invention relates to a method for development of a drug for treatment of a mast cell mediated inflammatory disorder in a mammal, most preferably a human. The method comprises using the anti-apoptotic bfl-1 gene or gene product as a molecular target. Alternatively, a bfl-1 homologue may be used, such as a member of the murine homologue family A1.

From U.S. 6,001,992 targeting pro-survival genes with antisense compounds is known. U.S. 5,843,773 discloses the sequence of blf-1 and the relation to cancer. Mast cell mediated inflammations are not disclosed.

Further, the invention also relates to a method of treatment of a mast cell mediated inflammatory disorder in a mammal, most preferably a human. The method comprises regulating the longevity and/or activity of activated mast cells by inhibiting the expression and/or function of the anti-apoptotic bfl-1 or A1 gene or gene product. In a preferred embodiment, the inhibition of the expression and/or function of the bfl-1 gene or gene product is obtained by targeting inflamed tissue in the affected mammal, preferably the human, with a drug that inhibits the function. Examples of compounds useful in the present method are as discussed above.

Detailed description of the drawings

Figure 1 illustrates the survival promotion of mast cells after cross-linkage of FcεRI (IgE CL). MCP5/L cells (A) or BMCMC from C57BL/6 (B and C) were either stimulated through crosslinkage of FcεRI or left untreated in RPMI medium deprived of serum and growth factors. Cell viability was determined by trypan blue exclusion and presented as the percentage of input cells that are still surviving when examined every 24 hours (A and B). In C, cell apoptosis was assessed by ELISA measuring the release of nucleosomes into the culture supernatant after 24 hours.

Figure 2 illustrates the survival promotion of human mast cells after cross-linkage of FcεRI (IgE CL). Survival of human CBCMC was assessed as explained in fig. 1. Inset indicates levels of nucleosome release shown in arbitrary units.

Figure 3 illustrates bcl-2 family gene regulation in mouse mast cells after cross-linkage of FcεRI (IgE CL). A, RPA was performed to analyze the expression levels of the indicated genes on total RNA isolated from MCP5/L cells either unstimulated or activated for 6 hours through FcεRI cross-linkage. Lane 1, untreated, normal cultured cells, Lane 2, control cells that were incubated and washed through the same procedures needed for FcεRI cross-linkage. Lane 3, cells that were stimulated through FcεRI cross-linkage. B, Phosphoimaging signals presented in A (lanes 2

and 3) are shown as gene expression relative to the average expression of the house keeping genes GAPDH and L32. Data were normalized such that the densitometric level of each gene from the control cells was given a value of 1.

Figure 4 illustrates bcl-2 family gene regulation in human mast cells after cross-linkage of FcεRI (IgE CL). RPA was performed to analyze the expression levels of the indicated genes on total RNA isolated from CBCMC either unstimulated or activated through FcεRI cross-linkage for 2, 6 and 12 hr (A) and phosphoimaging signals of the A1 gene (B) are densitrometrically analyzed as explained in Fig. 3.

Figure 5 illustrates the time-dependent up-regulation of A1 gene. RNA was isolated from MCP5/L cells activated through FcεRI crosslinkage for various time points as indicated and RPA was performed to analyze the kinetics of A1 induction.

Figure 6 illustrates regranulation of mouse mast cells. MCP5/L cells were activated through FcεRI cross-linkage up to 24 hours, the cells were then washed and incubated under normal culture condition. 48 hours after the first activation, cellular IgE receptors were cross-linked again. A1 induction at time points both in the firtst (1^{st} CL) and the second (2^{nd} CL) activation periods (A) and β-hexosaminidase (β-hex) release 30 min after cross-linkage (B) were assayed as explained in Fig 8 below.

Figure 7 illustrates the effects of ionomycin (A) and compound 48/80 (B) on A1 induction, release and survival of MCP5/L cells. RPA was performed on RNA derived from cells incubated for 6 hours in the presence of ionomycin or compound 48/80 at concentrations indicated (upper panels). Release of β-hexosaminidase (β-hex) after 30 min is shown (middle panels) as explained in Fig. 8. Survival rate of mast cells after 4 days in medium deprived of serum and growth factors was measu-

incubation (C), were determined as a measurement of activation and de-granulation by an enzymatic colorimetric assay.

Figure 9 illustrates the effects of inhibitors on induction of A1. RPA was performed on RNA derived from MCP5/L cells stimulated for 6 hr through FcεRI cross-linkage in the presence of various inhibitors as indicated. Densitometric analyses are shown as the A1/L32 ratio. A1 induction for activated cells without inhibitors was taken as 100.

Figure 10 illustrates the effect of cyclosporin A (CSA) on the survival promotion of mast cells through FcεRI cross-linkage. MCP5/L cells were either stimulated through FcεRI or left untreated for 5 days in medium deprived of serum and growth factors. Viability was determined by trypan blue exclusion.

Figure 11 illustrates the absence of survival promotion of mast cells from $A1^{-/-}$ mice after FcεRI cross-linkage (IgE CL). A, β-hexosaminidase (β-hex) release from $A1^{-/-}$ mast cells was assessed as explained in Fig. 6 to show that the cells were activated and released β-hexosaminidase. B, BMCMC from A1 knock-outs were stimulated and viability was determined as explained in Fig. 1B.

Figure 12 illustrates the accumulation of A1 protein in resting and activated (IgE-CL) BMCMC analyzed by western blot analysis.

Figure 13 A-B illustrates the effects of various inhibitors on bfl-1 induction in human mast cells after activation through FcεRI. A, RPA was performed on RNA derived from resting or activated (IgE-CL) CBCMC for 2 hr in the presence of various inhibitors (WTN= wortmannin; DEX=dexamethasone; PIC=piceatannol or EDTA). Cells were also activated by treatment with ionomycin (ION) for 2 hr. B, densitometric analysis in arbitrary units are shown as the bfl-1/L32 ratio. bfl-1 constitutive expression was taken as 1.

Figure 14 A-B illustrates that IgE-CL but not adenosine induce expression of A1 and mast cell survival. Mouse mast cells were activated either through cross-linkage of FcεRI or by treatment with adenosine (1 μM). A, RPA was perfomed on RNA from resting and activated mast cells. B, mast cell survival was determined as explained in figure 1.

EXPERIMENTAL

To investigate the role of bfl-1 or A1 expression and function for the longevity of activated mast cells, a number of experiments were performed using mouse and human mast cells. It is to be understood that the present examples are only provided for illustrative purposes and are not to be construed as limiting the invention as defined by the appended claims. All references given below and elsewhere in the present application are included herein by reference.

Materials and methods

Mast cell cultures:

Bone marrow-derived cultured mouse mast cells (BMCMC) were obtained by culturing mouse (C57BL/6 or $A1^{-/-}$ [Hamasaki, A.. F. Sendo, K. Nakayama, N. Ishida, I. Negishi, K, Nakayama and S. Hatakeyama. 1998. Accelerated neutrophil apoptosis in mice lacking A1-a, a subtype of the bcl-2-related A1 gene. *J Exp Med* 188:1985-92.], 4- to 5-week old,) bone marrow cells for 5 to 6 weeks in 10% WEHI-3 (IL-3 producing cell-line) enriched conditioned RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL, Germany), 4 mM L-glutamine, 50 µm 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) 1 mM sodium pyruvate (Sigma), 0.1 mM non-essential amino acids (Sigma), 10 mM Hepes (Sigma), and 100 µg/ml penicillin/streptomycin. The mast cell differentiation was confirmed by toluidine blue staining. The growth factor dependent mouse mast cell line MCP5/L was maintained in the same medium as described above. For cell viability assays, the cell suspension was mixed with the vital dye, trypan blue, and only the number of live cells was scored.

Cord blood cultured human mast cells (CBCMC) were differentiated from cord blood cells cultured for 8-10 weeks in 50 ng/ml SCF and 10 ng/ml IL-6 (Pepro Tech, London UK) and complete RPMI medium. The purity of human mast cells was >95% by tryptase staining.

Activation of mast cells *in vitro*:

For FcεRI-dependent activation, MCP5/L were resuspended at 1 x 10^6 cells/ml and sensitized using a monoclonal murine IgE anti-TNP (trinitrophenyl) (IgEl-b4, ATCC, Manassas, VA) antibody (15% hybridoma supernatant) for 90 min. The cells were washed twice with warm medium, followed by challenge with 1 μg/ml TNP-BSA for time periods indicated. For cell viability assays, both the antibody sensitization and the antigen challenge were performed in RPMI medium supplemented with 0.5 % BSA (Sigma) at 37 °C in a humidified incubator containing 5% CO_2. For gene regulation studies, all the incubations were carried out in RPMI medium supplemented with 5% fetal bovine serum and antibiotics on a rocker platform. In some experiments, cells were cross-linked in the medium containing 1mM EDTA or cells were incubated in ice-cold water during FcεRI cross-linking. Where indicated, various inhibitors were introduced to test their effects on A1 regulation after FcεRI cross-linking. These include bisindolylmaleimid (100 nM), wortmannin (100 nM), SB 203580 (pyridinylimidazole compound, inhibitor of p38 mitogen-activated protein kinase) (1 μM), PD 98059 (2- (2-amino-3-methoxyphenyl)-4-oxo-4H-[1]benzopyran, inhibitor of mitogen-activated protein kinase Kinase-1 (MEK1) (10μM), genistein (1 μM), suramin (100 μM), cyclosporin A (2 μg/ml), dexamethasone (1 μM), piceatannol (50 μg/ml) and cycloheximide (10 μg/ml). Dexamethasone and suramin were added 14 hr and 24 hr, respectively, before IgE sensitization. All the other inhibitors were added at the same time as FcεRI aggregation. In some A1 regulation assays, cells were resuspended in medium containing calcium ionophores (ionomycin), compound 48/80 (N-methyl-p-methoxyphenetylamine) and adenosine as well as cytokines including IL-3, IL-4, SCF, GM-CSF (granulocyte-macrophage colony stimulating factor) and TNF-α (tumor necrosis factor) and incubated for 6 hr at 37 °C on a rocker platform. After various treatments mentioned above, cell pellets were collected and kept at – 80 °C.

Human myeloma IgE (ND) and mouse anti-human IgE (clone 346) were used to activate CBCMC through cross-linkage of the high-affinity IgE-receptor. Human

mast cells at 1 x 10^6 cells/ml were incubated with 1 μg/ml IgE overnight, washed twice with PBS, plated at 3 x 10^5 cells/ml, and challenged with 2.0 μg/ml anti-IgE for 30 min at 37 °C.

Cell viability measurement:

Trypan blue exclusion assay was used in all of the cell survival experiments. In brief, cell suspension was mixed with the vital dye, trypan blue, and the number of live cells was scored. In some cases, cell apoptosis was also measured by Cell Death Detection ELISA (Boehringer Mannheim, Mannheim, Germany) quantitatively detecting the mono- and oligonucleosomes released into culture supernatant.

Western blot analysis

BMCMC were activated through FcεRI cross-linkage. Cells were harvested at 6, 12, 18, 30, 48 and 72 hours postactivation, washed once in PBS and lysed in hot 2x SDS gel sample buffer (20 mM dithiothreitol, 6% SDS, 0.25 M Tris, pH 6.8, 10% glycerol, and bromophenyl blue). The protein concentration in the samples was measured using a Bradford assay (Bio-Rad, Hercutes, CA, USA) and 50 μg of protein was resolved on SDS-10% polyacrylamide gels. Protein was then electroblotted to nitrocellulose membrane. Equal protein loading was confirmed by Ponceau S (Sigma) staining of the gel after transfer. A1 protein was detected using a rat monoclonal antiserum against murine A1 (1.0 μg/ml) (R & D Systems, Minneapolis, MN, USA), followed by a horseradish peroxidase conjugated sheep anti-rat antibody (Amersham, Life Sciences, Buckinghamshire, England) at a 1:2000 dilution. Western blots were then visualised by chemoluminescence using ECL.

Isolation of RNA and RNAse Protection Assay (RPA) analysis:

Total cellular RNA was isolated using the TriPure isolation reagent (Boehringer Mannheim, Mannheim, Germany). RPA was performed using the mAPO-2 and hAPO-2 multi-probe set from the RiboQuant System (PharMingen, San Diego, CA) following the supplier's recommended protocol. Briefly, 10 μg of RNA was hybri-

dized overnight at 56 °C with the ^{32}P-labelled probes synthesized from the mAPO-2/h-APO-2 multi-probe template set. Protected fragments were precipitated and size fractionated by 5% polyacrylamide gels. Pixel intensity was determined using a phosphoimaging device. Levels of each gene transcript were quantified by MacBas V2.2 (Fuji Photo Film Co. Ltd., Japan).

Measurement of release:

For detection of the granule-associated N-acetyl-β-D-hexosaminidase (β-hexosaminidase), an enzymatic colorimetric assay was used. Supernatant or a combination of supernatant and cell lysate was mixed with identical volume of substrate solution (7.5 mM p-nitrophenyl N-acetyl-β-D-glucosaminide (Sigma) in 80 mM citric acid, pH 4.5), and incubated at 37 °C for 2 hr. The reaction was stopped by adding glycine (0.2 M, pH 10.7) into each well and the absorbance was measured. Percentage of β-hexosaminidase release is calculated as the absorbance of the supernatant over that of the combination of supernatant and lysate.

Statistical analysis

Statistics were calculated using an analysis of variance (ANOVA), followed by multiple comparison using Fisher's method. * denotes $p<0.05$, ** $p<0.01$. Values presented are the means ± SEM.

Results

IL-3 is one of the primary growth factors for mast cells. MCP5/L is a growth factor-dependent mouse mast cell line, which requires the inclusion of IL-3 in its medium for survival and proliferation. First it was determined whether mast cells undergo apoptosis upon withdrawal of WEHI conditioned medium, (which forms the source of IL-3). As Shown in Fig. 1A, withdrawal of WEHI from MCP5/L mast cells resulted in a progressive decrease in the number of live cells as determined by trypan blue exclusion staining. To examine whether mast cell activation would influence this apoptotic process following withdrawal of growth factor, MCP5/L cells were

activated through FcεRI aggregation. As also shown in Fig. 1, percentage cell survival of activated cells, demonstrated by trypan blue exclusion, increased as compared with control cells after 2 days of incubation.

On day 4 and 5, FcεRI aggregation caused cell survival to increase about 70% and 120%, respectively, over control cells.

Similar findings were obtained using mouse BMCMC (Fig. 1B) and human CBCMC (Fig. 2) although withdrawal of exogenous growth factors resulted in a more rapid decrease in viability as compared with the mouse cell line. Two days following the IgE crosslinkage, survival rate of the activated cells surpassed that of the control cells by 400% and 250%, respectively, for BMCMC (Fig 1B) and CBCMC (Fig 2). By measuring release of mono- and oligo-nucleosomes we could confirm that cross-linkage of FcεRI rescued the cells from undergoing apoptosis (Fig. 1C and Fig. 2, inset). Thus, activation of mast cells through the high-affinity IgE-receptor initiates a cellular response that directly prevents apoptosis, without addition of growth factors.

To determine whether mast cells transcribe any of the apoptosis-related genes constitutively or following activation (FcεRI aggregation), RPA and densitometric analysis were performed. By using the mAPO-2 multi-probe set from PharMingen, the expression pattern of murine bcl-2 family genes were analyzed (Fig. 3). A striking up-regulation of the pro-survival bcl-2 homologue A1 was observed, following FcεRI aggregation for 6 hr. Serum starvation and growth factor withdrawal could not induce A1 expression (Fig. 3A). For other genes analyzed, no obvious regulation was seen directly from the densitrometric analyses. The expression of A1 in mast cells was confirmed using C57 and M9/9, two other murine mast cell lines, and BMCMC (data not shown).

Similarly, hAPO-2 multi-probe set was used to analyse the human bcl-2 family gene regulation. A strong induction of the human homologue of murine A1, bfl-1, was

found, following 2 hr of FcεRI activation (Fig. 4A) and the quantitative densitometric analyses showed an up-regulation of the gene by over 100-fold as compared with control cells (Fig. 4B).

RPA was also performed to determine the kinetics of A1 expression. Cells were sensitized with monoclonal IgE for 90 min before FcεRI aggregation with TNP-BSA. Cells were harvested at the time points indicated (Fig. 5).
Following FcεRI aggregation, A1 RNA was first detected at 2 hr, peaked around 5 hr, and was no longer detectable by 24 hr. Expression of A1 protein could be determined 6 hr after activation, peaked around 48 hr, and then decliend (Fig. 12).

In contrast to mouse cells, bfl-1 in CBCMC was both induced and declined more rapidly (Fig. 4A).

As seen in Fig. 5, A1 expression following FcεRI aggregation peaked by 6 hr and dissappeared after 18 to 24 hr. Under physiological conditions, mast cells do not die after degranulation triggered by FcεRI aggregation and they can be activated again. Therefore it was tested whether, after the first activation, A1 could be induced again by a second FcεRI aggregation. 24 hr after the first activation, cells were washed, maintained in complete culture medium for another 24 hr, and then activated again by FcεRI FcεRI aggregation. Similar with the kinetics of a single activation (Fig. 5), A1 appeared 2 hr after the second FcεRI aggregation, and peaked around 6 hr (Fig. 6).

To determine whether the regulation of A1 transcript was only seen with FcεRI aggregation as a physiologic stimulus, mast cells were also exposed to ionomycin and C48/80 for 6 hr. As shown in Fig. 7A, ionomycin also induced expression of A1 and promoted mast cell survival. In contrast, C48/80 at concentrations of up to 100 μg/ml has no effect on A1 gene transcription (Fig. 7B). Under the concentrations used, C48/80 triggered substantial β-hexosaminidase release but the cells did not

survive. Another endogenous mast cell secretagogoues, adenosine, was also tested to investigate if activation through the adenosine receptor would induce A1 expression and promote mast cell survival. As seen in figure 14, no induction of A1 could be determined, and no increased survival in adenosine activated mast cells was obtained, although the cells released significant amounts of β-hexosaminidase (data not shown).

In efforts to gain insight as to whether A1 expression is mediated by calcium influx following mast cell activation, it was determined whether A1 was up-regulated upon FcεRI aggregation in the presence of EDTA or when the cells were incubated in ice-cold water. Remarkably, both EDTA and ice-cold water treatments blocked A1 expression induced by FcεRI aggregation (Fig. 8A). EDTA can block the calcium mobilization of mast cells and thereby inhibit release of mast cell granular enzyme β-hexosaminidase following FcεRI aggregation (Fig 8B). Similarly, when incubated in ice-cold water, β-hexosaminidase release was also inhibited (Fig. 8C).

The effects on A1 induction of a number of inhibitors that are directed against various signal transduction pathways were also tested and densitometric analysis was performed (Fig. 9). Wortmannin, a PI-3 kinase inhibitor, cyclosporin A, an immunosuppressive agent, and cycloheximide, an inhibitor of *de novo* synthesis of early gene products, and dexamethasone, a glucocorticoid, could completely block A1 induction. Similar effects were obtained when human mast cells were treated with wortmannin, dexamethasone and EDTA. In addition, human mast cells were also treated with the Syk inhibitor Piceatannol, which totally inhibited the induction of A1 (Figure 13).

It was further tested whether CSA could block the survival promotion of mast cells triggered by FcεRI crosslinkage. CSA did not affect control MCP5/L apoptosis. However, when cells were activated through IgE receptor crosslinkage, addition of CSA dramatically reduced the number of live cells (Fig. 10).

To address the issue whether A1 is a prerequisite for mast cell survival upon cross-linkage of FcεRI, or if the up-regulation of A1 is just a parallel phenomenon, mast cells deficient in A1 were used. Upon activation with IgE and antigen the $A1^{-/-}$ BMCMC released granule associated β-hexosaminidase similar to wild-type control (Fig. 11A). Cross-linkage of FcεRI did not promote survival of these cells, instead they died at the same rate as resting control cells (Fig. 11B). This finding confirms the hypothesis that A1 expression is necessary for mast cell survival upon allergic activation.

In the present invention the pro-survival gene bfl-1 or A1 has been identified to be crucial for mast cell survival after activation of mast cells through the high affinity IgE receptor. Interfering with the transcriptional regulation, the expression of bfl-1 or A1, the activity of the bfl-1 or A1 protein, or its interaction with pro-survival genes can be used to prevent mast cells to survive an allergy associated activation. The present invention provides a method to develop drugs and to treat various diseases in mammals, most preferably humans, where a local activation of mast cells are of importance for the symptoms. Thus it can be used to treat e.g. nasal mucosa in hay fever, respiratory tract in asthma, the skin in atopic dermatitis, the gastrointestinal tract in food allergy etc. The resulting drug of the invention which could be a protein, a (poly)peptide, oligonucleotide, or an organic molecule is delivered in a manner consistent with the conventional methods associated with the treatment of the particular disease such for example orally, intravenously, intramuscularly, topically, subcutaneously or by aerosol inhalant in order to prevent the inflammatory reactions associated with such diseases.

CLAIMS

1. Use of a compound capable of suppressing the expression of the human bfl-1 gene or a homologue thereof in the manufacture of a medicament for treating a mast cell mediated inflammatory disorder in a mammal by negatively regulating the longevity and/or activity of activated mast cells.
2. Use of a compound capable of suppressing the function of a transcriptional product of the human bfl-1 gene or a homologue thereof in the manufacture of a medicament for treating a mast cell mediated inflammatory disorder in a mammal by negatively regulating the longevity and/or activity of activated mast cells.
3. Use of a compound capable of suppressing the function of a translational product of the human bfl-1 gene or a homologue thereof in the manufacture of a medicament for treating a mast cell mediated inflammatory disorder in a mammal by negatively regulating the longevity and/or activity of activated mast cells.
4. Use according to any one of claims 1-3, wherein the compound is capable of suppressing the bfl-1 dependent survival of activated mast cells.
5. Use according to any one of claims 1-4, wherein the compound is a peptide, polypeptide, oligonucleotide or any other organic molecule.
6. Use according to any one of claims 1-4, wherein the compound is cyclosporin A.
7. Use according to any one of claims 1-4, wherein the compound is a PI-3 kinase inhibitor, such as wortmannin.
8. Use according to any one of claims 1-4, wherein the compound is a glucocorticoid, such as dexamethasone.
9. A pharmaceutical composition, which comprises a compound capable of suppressing the expression of the human bfl-1 gene or a homologue thereof together with a pharmaceutically acceptable carrier.
10. A pharmaceutical composition, which comprises a compound capable of suppressing the function of a transcriptional product of the human bfl-1 gene or a homologue thereof together with a pharmaceutically acceptable carrier.

11. A pharmaceutical composition, which comprises a compound capable of suppressing the function of a translational product of the human bfl-1 gene or a homologue thereof together with a pharmaceutically acceptable carrier.

12. A pharmaceutical composition according to any one of claims 9-11, wherein said compound is selected from the group consisting of cyclosporin A, a PI-3 kinase inhibitor, and glucocorticoid.

13. A method of biological assay for identifying a compound for treatment of a mast cell mediated inflammatory disorder in a mammal, wherein the human bfl-1 gene or a homologue thereof is used as a molecular target.

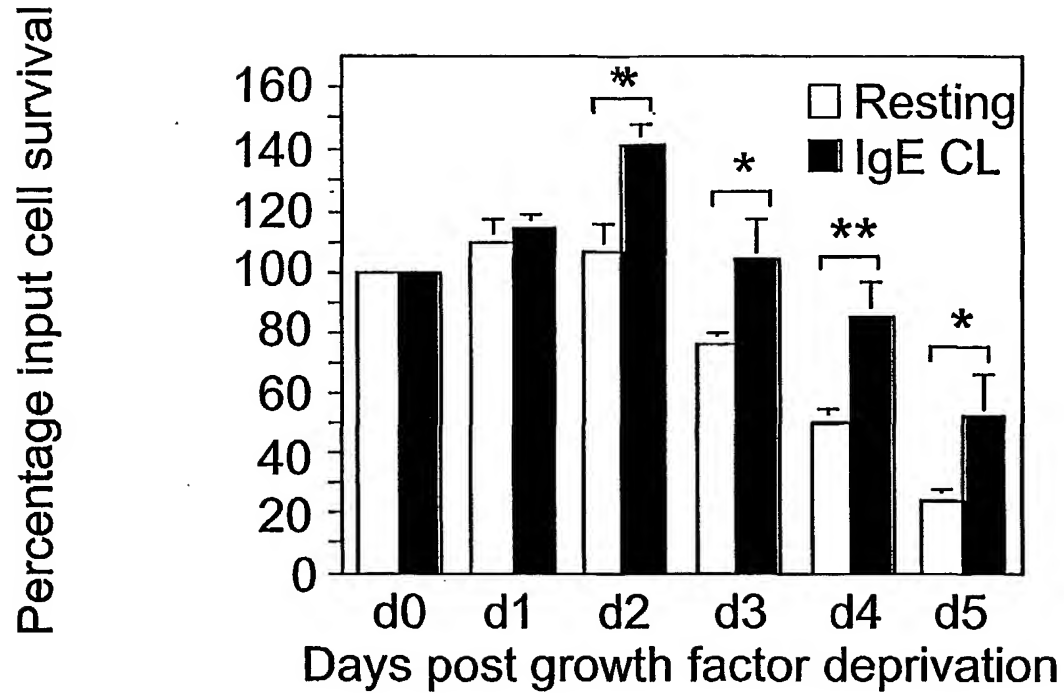
14. A method of biological assay for identifying a compound for treatment of a mast cell mediated inflammatory disorder in a mammal, wherein an expression product of the human bfl-1 gene or a homologue thereof is used as a molecular target.

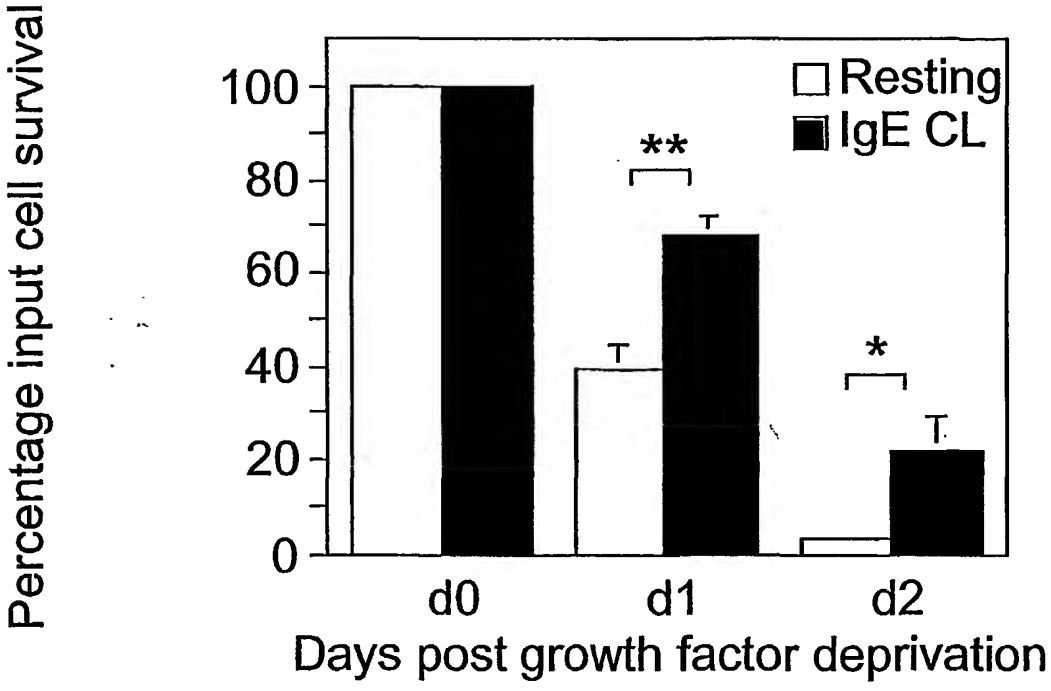
15. A method of chemical assay for identifying a compound for treatment of a mast cell mediated inflammatory disorder in a mammal, wherein the human bfl-1 gene or a homologue thereof is used as a molecular target.

16. A method of chemical assay for identifying a compound for treatment of a mast cell mediated inflammatory disorder in a mammal, wherein an expression product of the human bfl-1 gene or a homologue thereof is used as a molecular target.

17. A method for development of a drug for treatment of a mast cell mediated inflammatory disorder in a mammal, wherein the human bfl-1 gene or a homologue thereof is used as a molecular target.

18. A method for development of a drug for treatment of a mast cell mediated inflammatory disorder in a mammal, wherein an expression product of the human bfl-1 gene or a homologue thereof is used as a molecular target.


Percentage input cell survival
160
140
120
100
80
60
40
20
0
**
*
**
*
Resting
IgE CL
d0
d1
d2
d3
d4
d5
Days post growth factor deprivation


Percentage input cell survival
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60
40
20
0
**
*
Resting
IgE CL
d0
d1
d2
Days post growth factor deprivation

Fig. 1A

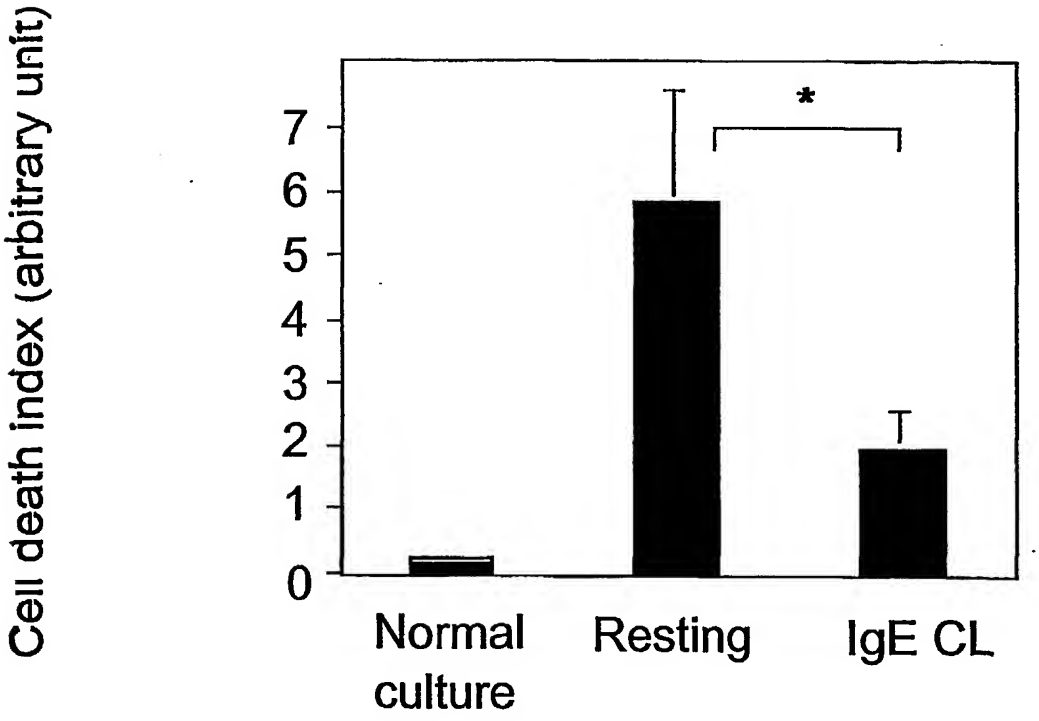

Cell death index (arbitrary unit)
0
1
2
3
4
5
6
7
*
Normal culture
Resting
IgE CL

Fig. 1B

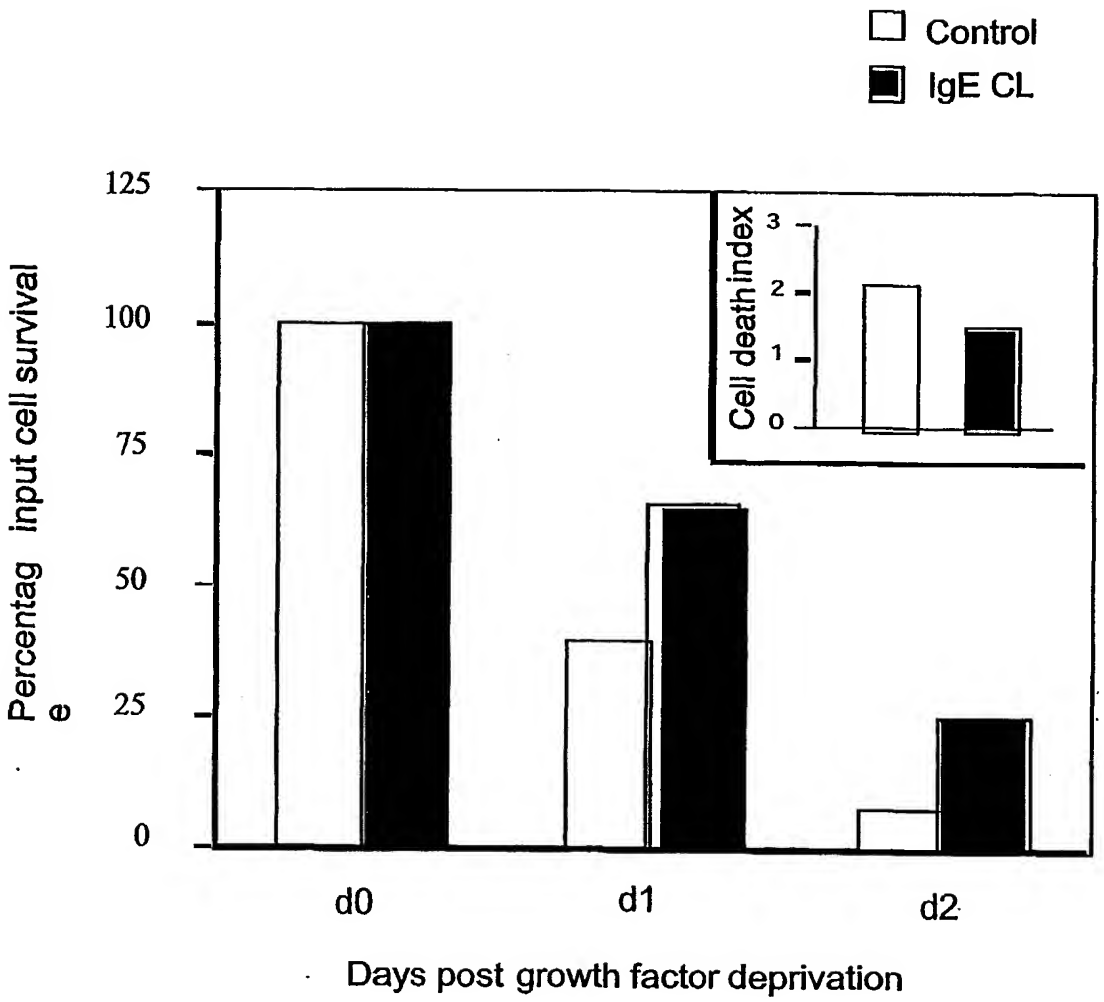
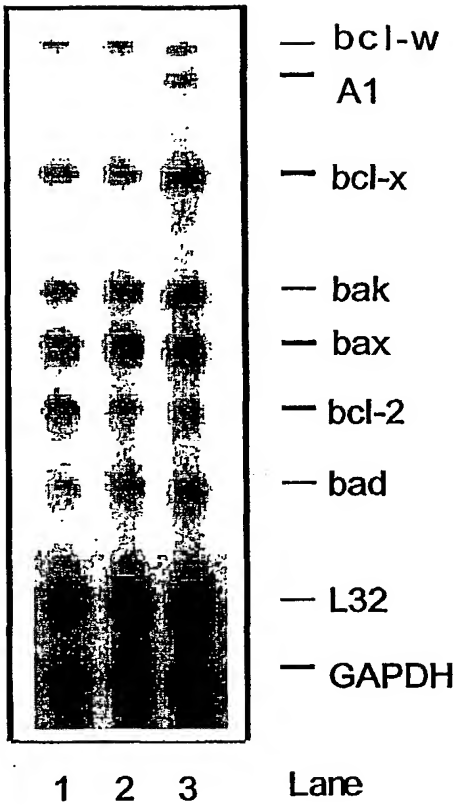

Control
IgE CL
Percentage input cell survival
125
100
75
50
25
0
d0
d1
d2
Days post growth factor deprivation
Cell death index
3
2
1
0

Fig. 2


bcl-w
A1
bcl-x
bak
bax
bcl-2
bad
L32
GAPDH
1 2 3
Lane

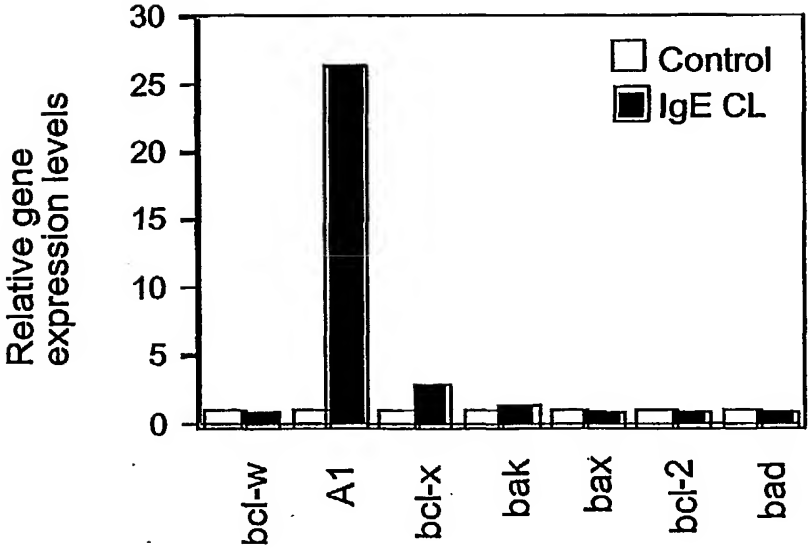

Relative gene expression levels
30
25
20
15
10
5
0
Control
IgE CL
bcl-w
A1
bcl-x
bak
bax
bcl-2
bad

Fig. 3

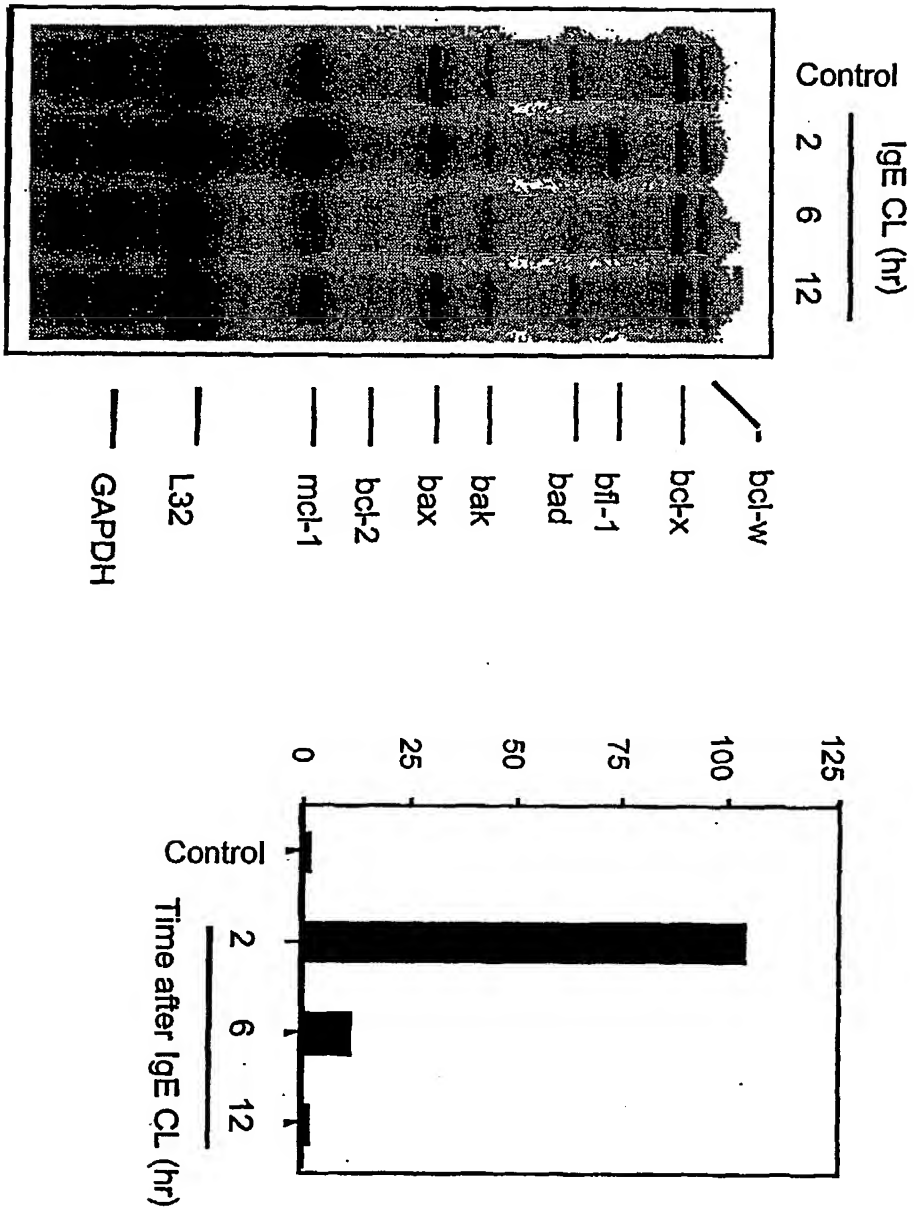

Control
IgE CL (hr)
2
6
12
bcl-w
bcl-x
bfl-1
bad
bak
bax
bcl-2
mcl-1
L32
GAPDH
0
25
50
75
100
125
Control
Time after IgE CL (hr)
2
6
12

Fig. 4

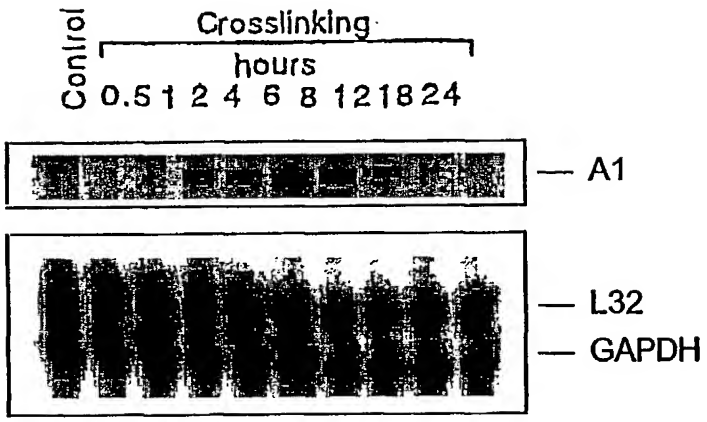
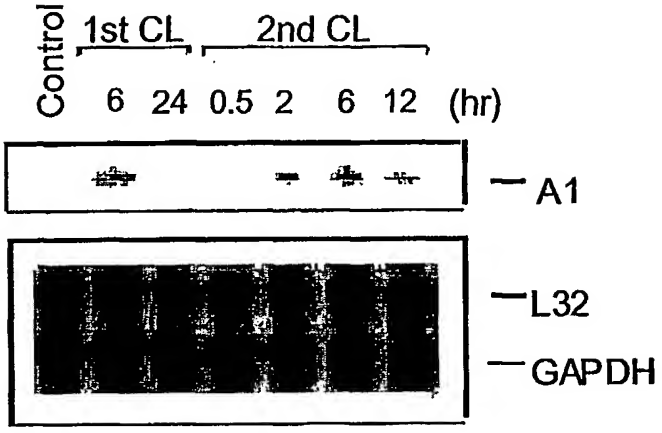


Fig.5


Control
1st CL
2nd CL
6 24 0.5 2 6 12 (hr)
A1
L32
GAPDH

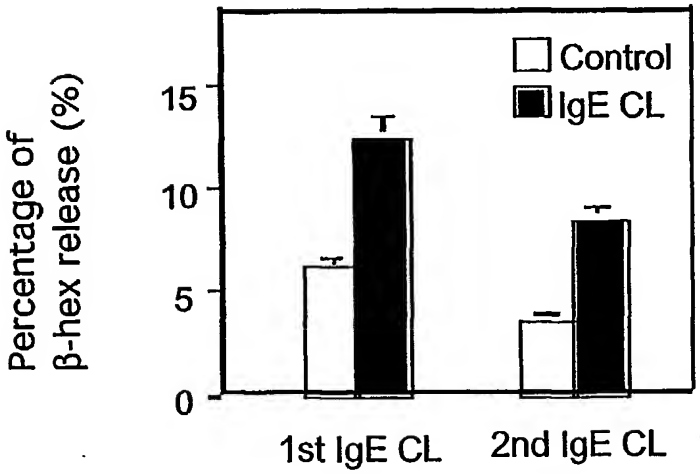

Percentage of β-hex release (%)
15
10
5
0
Control
IgE CL
1st IgE CL
2nd IgE CL

Fig. 6

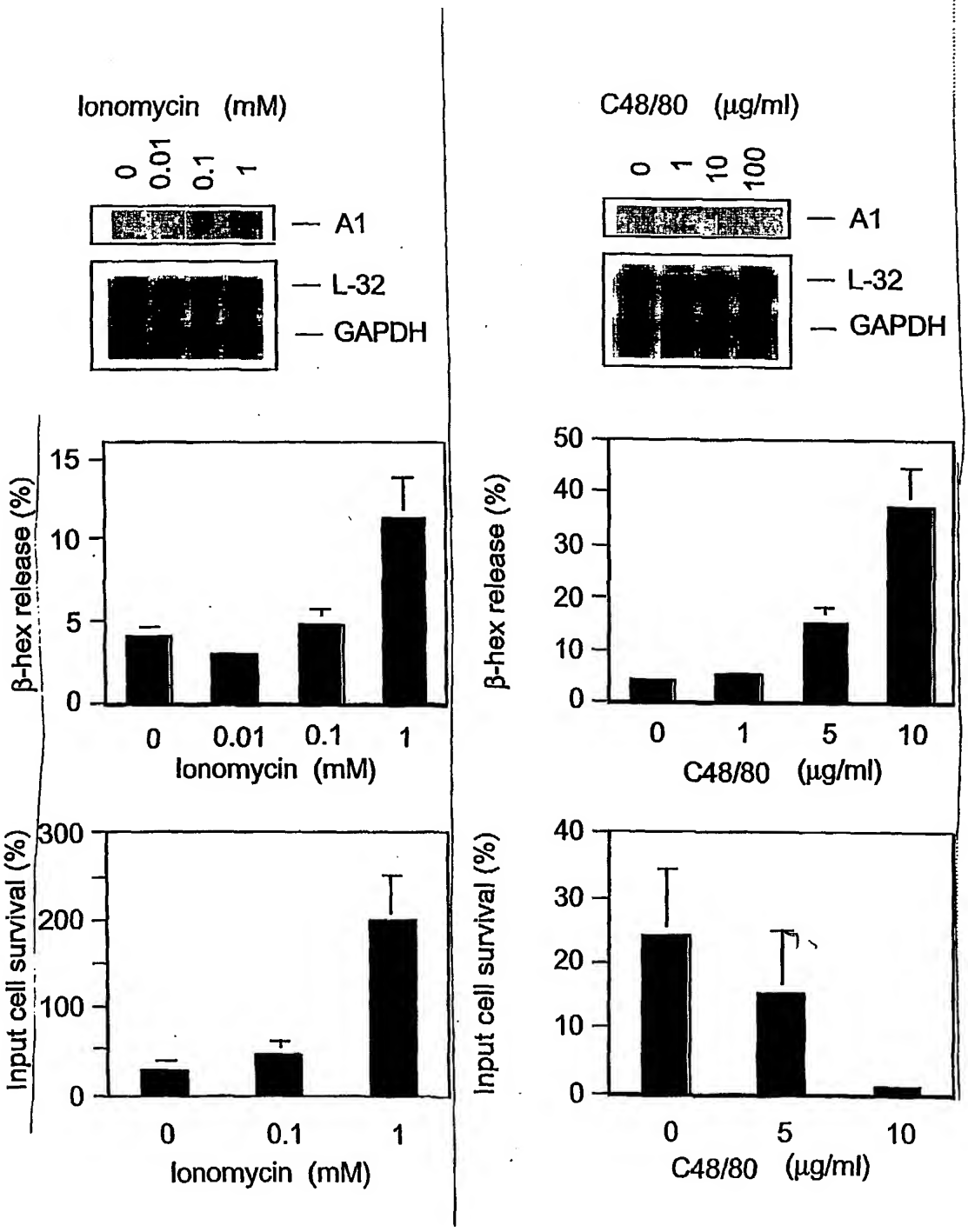

Ionomycin (mM)
0
0.01
0.1
1
A1
L-32
GAPDH
C48/80 (µg/ml)
0
1
10
100
A1
L-32
GAPDH
β-hex release (%)
15
10
5
0
0
0.01
0.1
1
Ionomycin (mM)
β-hex release (%)
50
40
30
20
10
0
0
1
5
10
C48/80 (µg/ml)
Input cell survival (%)
300
200
100
0
0
0.1
1
Ionomycin (mM)
Input cell survival (%)
40
30
20
10
0
0
5
10
C48/80 (µg/ml)

Fig.7

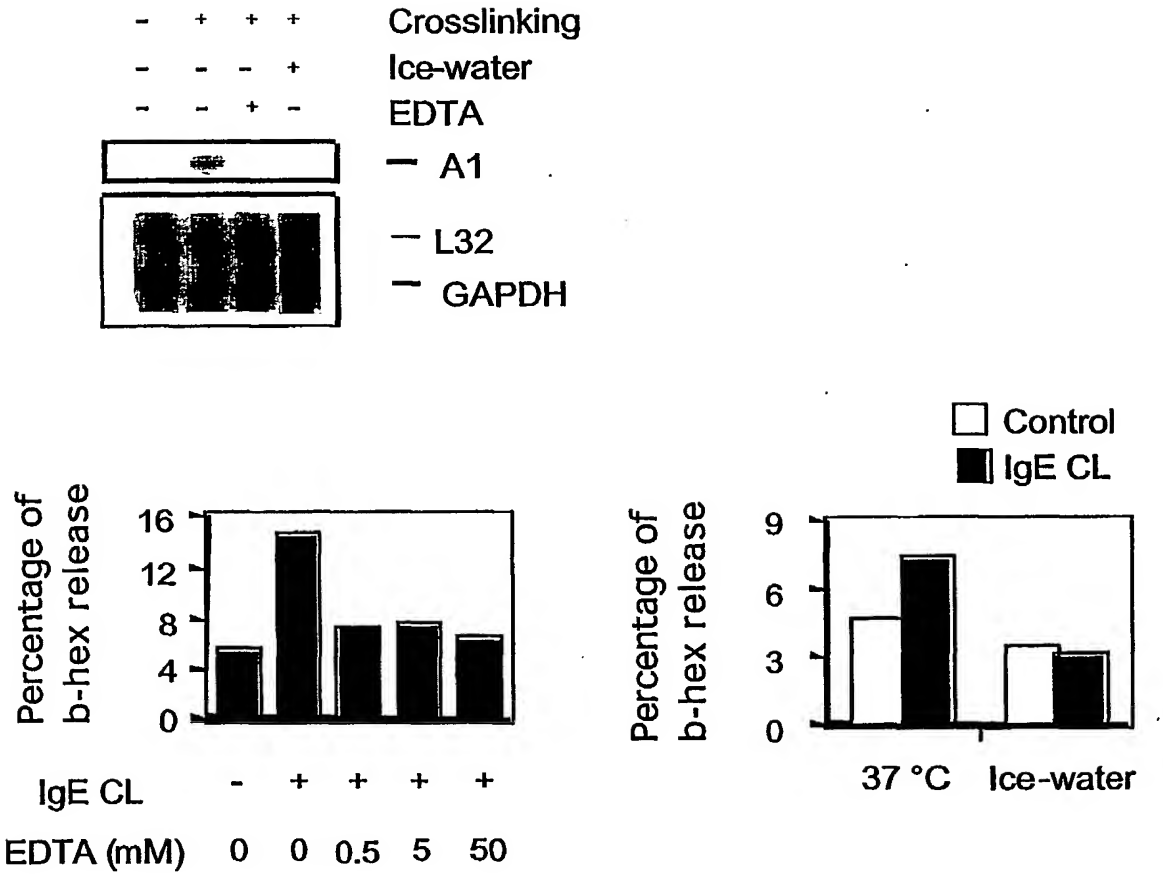

- + + + Crosslinking
- - - + Ice-water
- - + - EDTA
A1
L32
GAPDH
Percentage of b-hex release
16
12
8
4
0
IgE CL - + + + +
EDTA (mM) 0 0 0.5 5 50
Control
IgE CL
Percentage of b-hex release
9
6
3
0
37 °C
Ice-water

Fig. 8

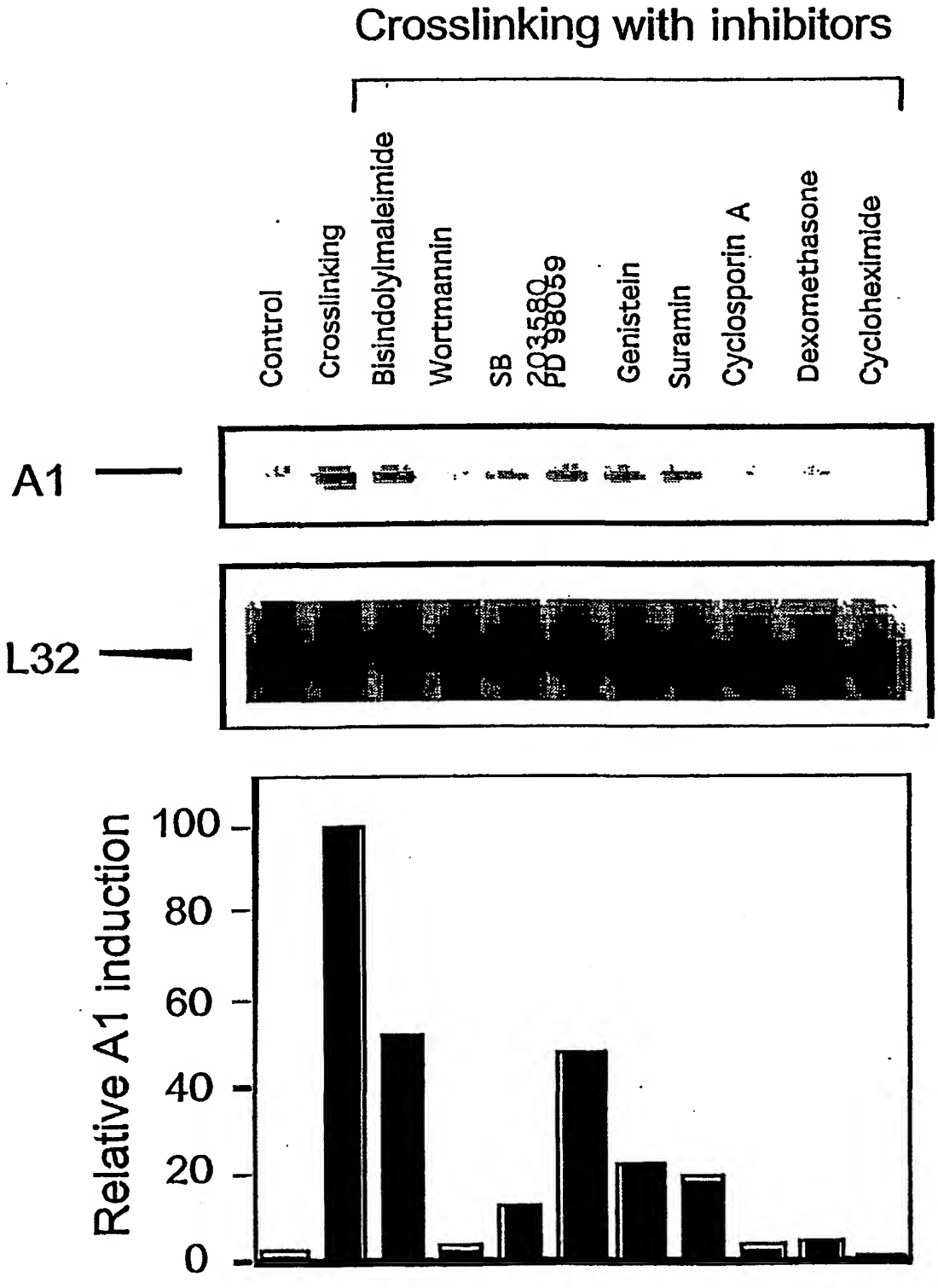

Crosslinking with inhibitors
Control
Crosslinking
Bisindolylmaleimide
Wortmannin
SB 203580
PD 98059
Genistein
Suramin
Cyclosporin A
Dexomethasone
Cycloheximide
A1
L32
Relative A1 induction
100
80
60
40
20
0
Densitometry

Fig. 9

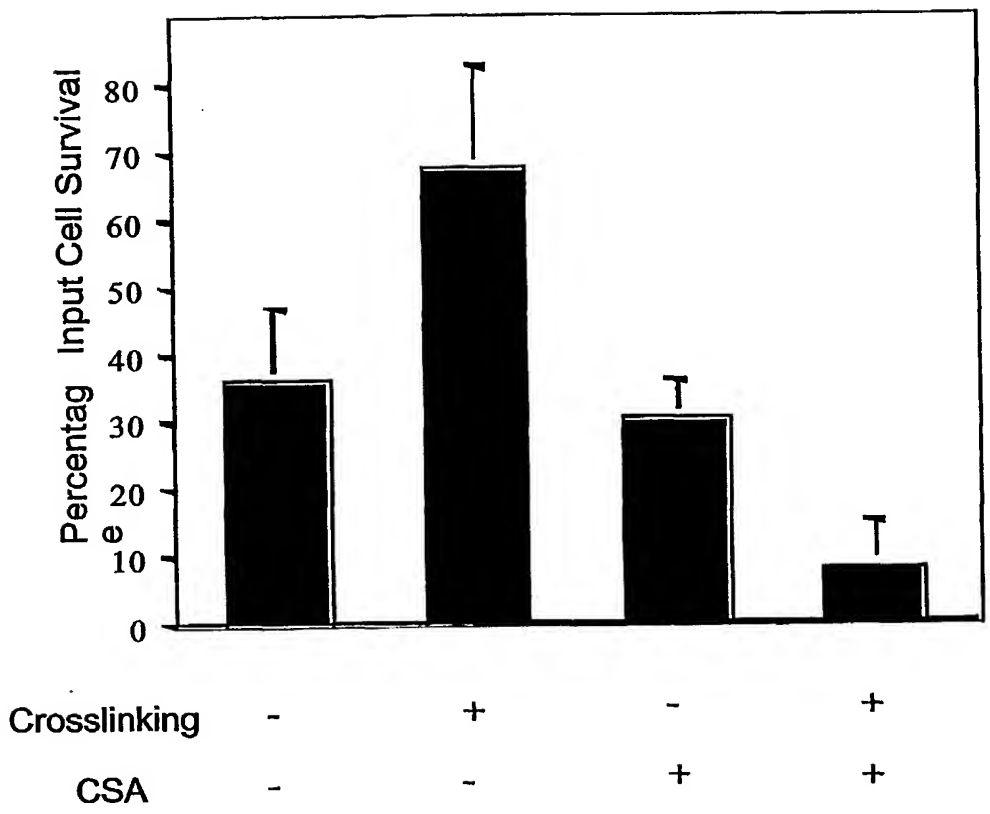

Percentage Input Cell Survival
80
70
60
50
40
30
20
10
0
Crosslinking - + - +
CSA - - + +

Fig. 10

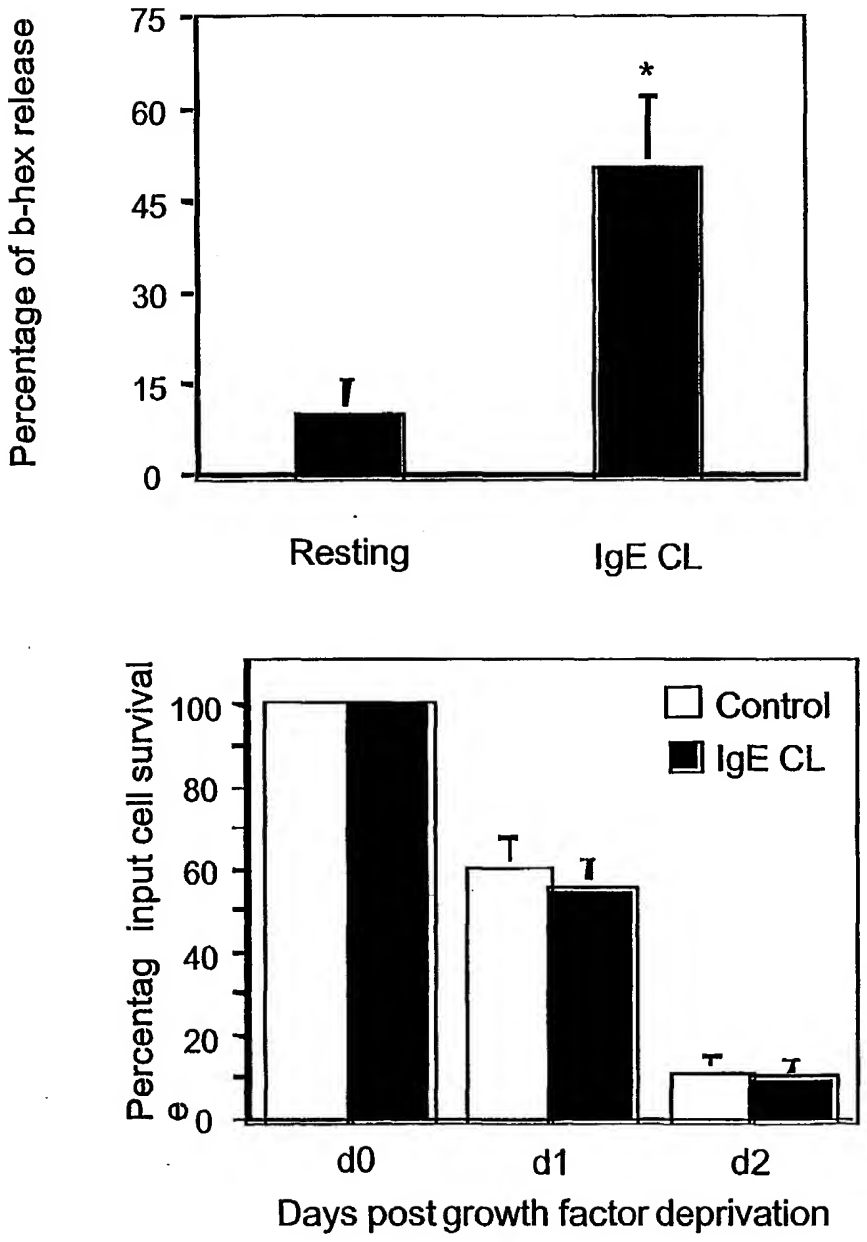

Percentage of b-hex release
75
60
45
30
15
0
*
Resting
IgE CL
Percentag input cell survival
100
80
60
40
20
0
Control
IgE CL
d0
d1
d2
Days post growth factor deprivation

Fig. 11

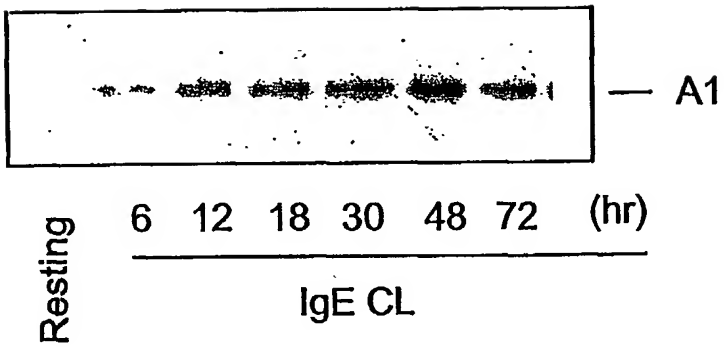


Fig. 12

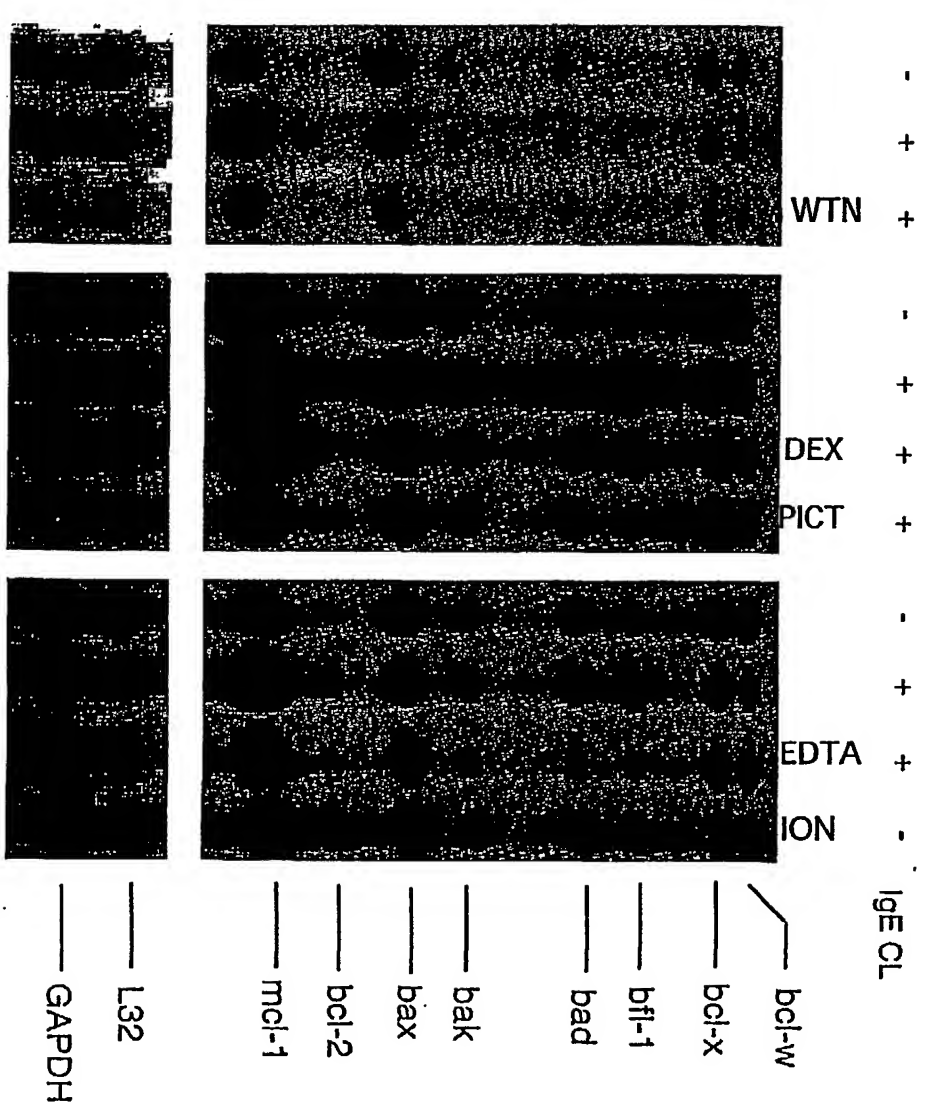

WTN
DEX
PICT
EDTA
ION
IgE CL
GAPDH
L32
mcl-1
bcl-2
bax
bak
bad
bfl-1
bcl-x
bcl-w

Fig.13A

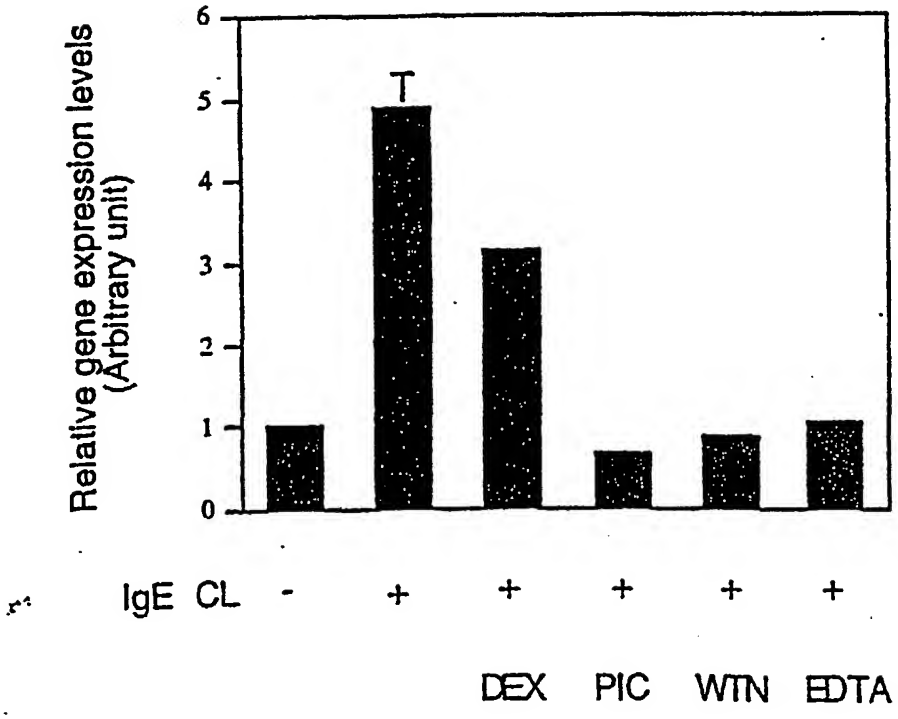

Relative gene expression levels (Arbitrary unit)
6
5
4
3
2
1
0
T
IgE CL - + + + + +
DEX PIC WTN EDTA

Fig. 13B

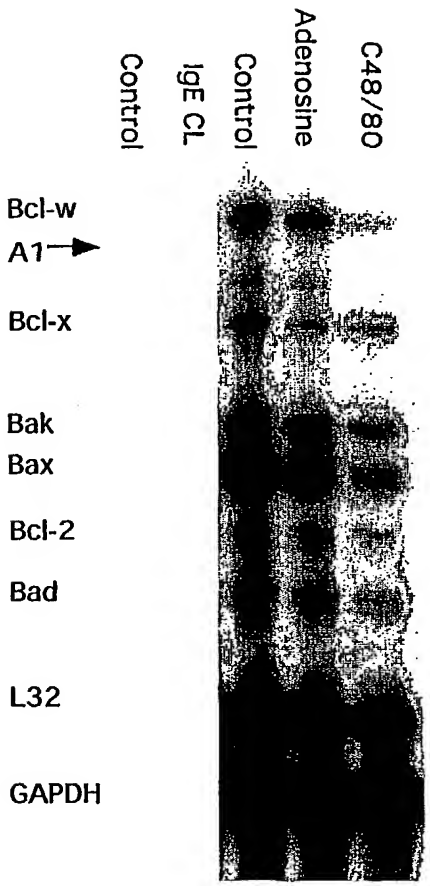

Control
IgE CL
Control
Adenosine
C48/80
Bcl-w
A1
Bcl-x
Bak
Bax
Bcl-2
Bad
L32
GAPDH

Fig. 14A

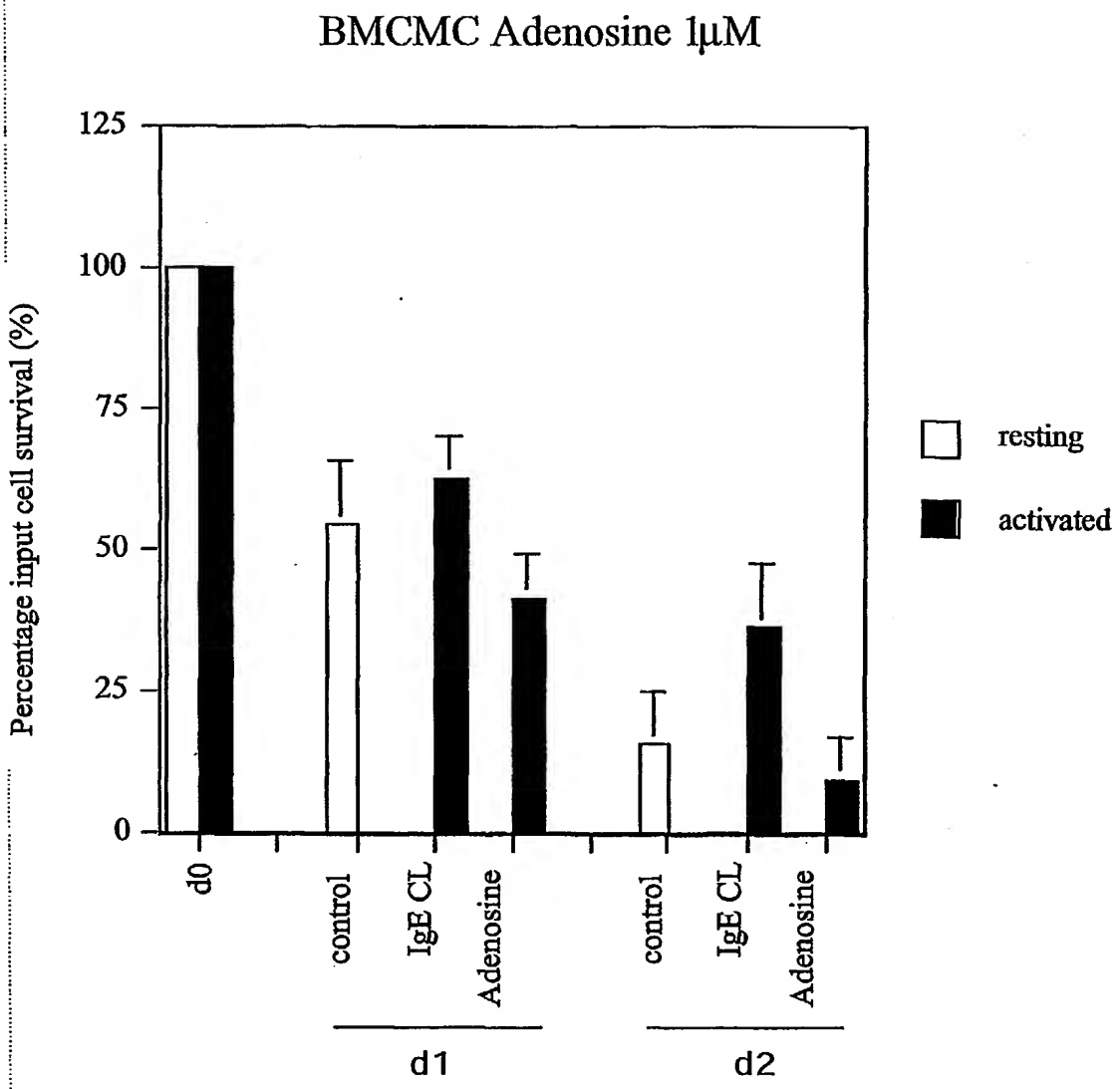

BMCMC Adenosine 1µM
Percentage input cell survival (%)
125
100
75
50
25
0
d0
control
IgE CL
Adenosine
control
IgE CL
Adenosine
d1
d2
resting
activated

Fig. 14B

SEQUENCE LISTING

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                                         1               5

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Tyr Ile Tyr Arg Leu Ala Gln Asp Tyr Leu Gln Cys Val Leu Gln Ile
        10                  15                  20

cca caa cct gga tca ggt cca agc aaa acg tcc aga gtg cta caa aat   151
Pro Gln Pro Gly Ser Gly Pro Ser Lys Thr Ser Arg Val Leu Gln Asn
    25                  30                  35

gtt gcg ttc tca gtc caa aaa gaa gtg gaa aag aat ctg aag tca tgc   199
Val Ala Phe Ser Val Gln Lys Glu Val Glu Lys Asn Leu Lys Ser Cys
 40                  45                  50                  55

ttg gac aat gtt aat gtt gtg tcc gta gac act gcc aga aca cta ttc   247
Leu Asp Asn Val Asn Val Val Ser Val Asp Thr Ala Arg Thr Leu Phe
                60                  65                  70

aac caa gtg atg gaa aag gag ttt gaa gac ggc atc att aac tgg gga   295
Asn Gln Val Met Glu Lys Glu Phe Glu Asp Gly Ile Ile Asn Trp Gly
            75                  80                  85

aga att gta acc ata ttt gca ttt gaa ggt att ctc atc aag aaa ctt   343
Arg Ile Val Thr Ile Phe Ala Phe Glu Gly Ile Leu Ile Lys Lys Leu
        90                  95                  100

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Leu Arg Gln Gln Ile Ala Pro Asp Val Asp Thr Tyr Lys Glu Ile Ser
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Tyr Phe Val Ala Glu Phe Ile Met Asn Asn Thr Gly Glu Trp Ile Arg
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caa aac gga ggc tgg gaa aat ggc ttt gta aag aag ttt gaa cct aaa   487
Gln Asn Gly Gly Trp Glu Asn Gly Phe Val Lys Lys Phe Glu Pro Lys
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tct ggc tgg atg act ttt cta gaa gtt aca gga aag atc tgt gaa atg   535
Ser Gly Trp Met Thr Phe Leu Glu Val Thr Gly Lys Ile Cys Glu Met
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cta tct ctc ctg aag caa tac tgt tga ccagaaagga cactccatat         582
Leu Ser Leu Leu Lys Gln Tyr Cys
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Thr Ser Arg Val Leu Gln Asn Val Ala Phe Ser Val Gln Lys Glu Val
         35                  40                  45
Glu Lys Asn Leu Lys Ser Cys Leu Asp Asn Val Asn Val Val Ser Val
     50                  55                  60
Asp Thr Ala Arg Thr Leu Phe Asn Gln Val Met Glu Lys Glu Phe Glu
 65                  70                  75                  80
Asp Gly Ile Ile Asn Trp Gly Arg Ile Val Thr Ile Phe Ala Phe Glu
                 85                  90                  95
Gly Ile Leu Ile Lys Lys Leu Leu Arg Gln Gln Ile Ala Pro Asp Val
            100                 105                 110
Asp Thr Tyr Lys Glu Ile Ser Tyr Phe Val Ala Glu Phe Ile Met Asn
        115                 120                 125
Asn Thr Gly Glu Trp Ile Arg Gln Asn Gly Gly Trp Glu Asn Gly Phe
    130                 135                 140
Val Lys Lys Phe Glu Pro Lys Ser Gly Trp Met Thr Phe Leu Glu Val
145                 150                 155                 160
Thr Gly Lys Ile Cys Glu Met Leu Ser Leu Leu Lys Gln Tyr Cys
                165                 170                 175
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